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FOREWORD

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N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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Table of Contents

Cover	i
SF 298	ii
Foreword	iii
Table of Contents	1
Introduction	2
Body	2
Key Research Accomplishments	7
Reportable Outcomes	7
Conclusions	7
References	7
Appendices	8

INTRODUCTION

Cell proliferation in the prostate is dependent upon androgen and androgen-induced proliferation is associated with specific changes in the cell cycle regulatory proteins. Testosterone has been shown to upregulate expression of certain cyclins and to downregulate cdk inhibitors. The purposes of this research are to examine the relation between cell cycle regulation and androgen-induced proliferation in prostate cancer, and secondly, to study the abnormalities in cell cycle regulation that accompany the transition of androgen-dependent prostate cancer to androgen-independence. Hereto, transferable human prostate tumors grown in the flanks of male nude mice are used as a model. This model allows for easy hormonal manipulation of tissue with human prostate histology, while the surrounding stroma which is from a different tissue and species has been shown not to affect tumor growth. The research focuses on characterization of the MDA Pca 2b cell line grown as a xenograft in nude mice. This cell line was established from a bone metastasis from a patient whose prostate cancer showed androgen-independent growth (Navone et al., 1997). Tumors were collected from intact mice and at various time points after castration and subsequent androgen replacement of tumor-bearing animals. Tissue was formalin fixed and the remainder was snapfrozen for protein isolation. Formalin-fixed tumor tissue was subjected to (immuno)histochemical analysis (hematoxylin-and-eosin, BrdU, TUNEL). Isolated proteins were subjected to western blot analysis for the presence of various positive and negative regulators of the G1 phase of the cell cycle.

BODY

Training Accomplishments

*As part of the research training the trainee instructed and supervised an undergraduate student who worked on a 10-week-project involving cell cycle alterations in the MDA Pca 2b human prostate cancer xenograft model.

*The 1999 Gordon Research Conference on Hormonal Carcinogenesis (Tilton, NH) was attended by the trainee. The trainee presented a poster on earlier work on cell kinetics in the mouse ventral prostate. The various seminars gave the trainee an overview of models used in prostate cancer research, their benefits and disadvantages. Furthermore, the conference provided an excellent opportunity to communicate with researchers in the prostate cancer field.

*Monthly meeting at the Department of Genitourinary Medical Oncology ceased to be organized, in lieu of these meetings monthly Prostate Meeting are being organized at the Science Park campus by the trainee. Faculty and postdocs from Science Park with interest in prostate research attend these meetings and present seminars on prostate-related topics. Minutes of these meetings are being written by the trainee.

*The trainee is involved in the ongoing research at Science Park with the K5-IGF1 mouse of Dr. DiGiovanni. Six-seven-month-old K5-IGF1 mice develop prostate cancer. This research has resulted in an PNAS publication co-authored by the trainee (DiGiovanni J, Kiguchi K, Frijhoff A, Wilker E, Bol DK, Beltran L, Moats S, Ramirez A, Jorcano J and Conti CJ. Deregulated expression of insulin-like growth factor I in prostate epithelium leads to neoplasia in transgenic mice. PNAS, 97(7):3455-60, 2000.)

*The trainee has received extensive training in grant writing. The trainee was involved in writing an NIH grant with Dr. M. Kazanietz, PI of the grant proposal, and Dr. Conti, the trainee's mentor. This grant has received a fundable score from the NCI. Furthermore, the trainee co-wrote together with Dr. Conti grant proposals for NIH and U.S. Army Medical research and Materiel Command.

*To gain a better understanding of prostate pathology the trainee audited the course "Introduction in Pathology", an undergraduate course taught by Dr. Gimenez-Conti at the Science Park Campus. The trainee passed finals with good results.

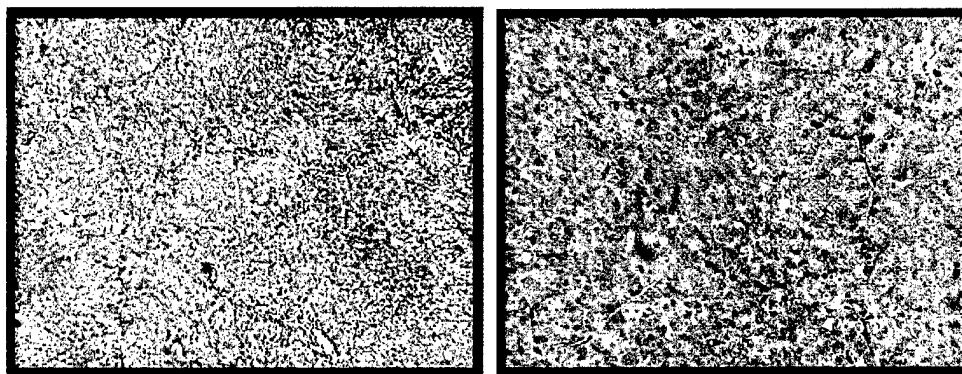


Fig. 1. Hematoxylin-and-eosin staining of MDA PCA 2b tumor grown in an intact nude mouse. Tissue was fixed in formalin, magnifications are (A) 10x, (B) 20x.

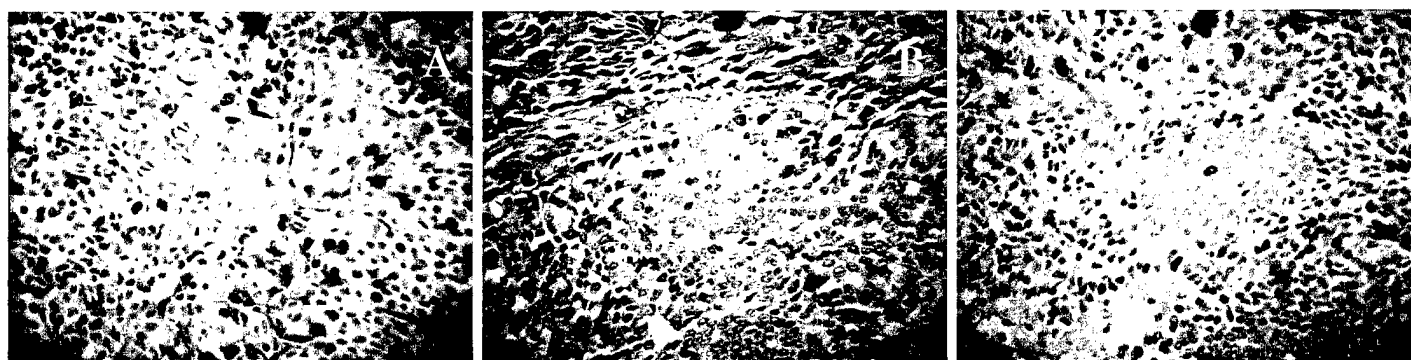
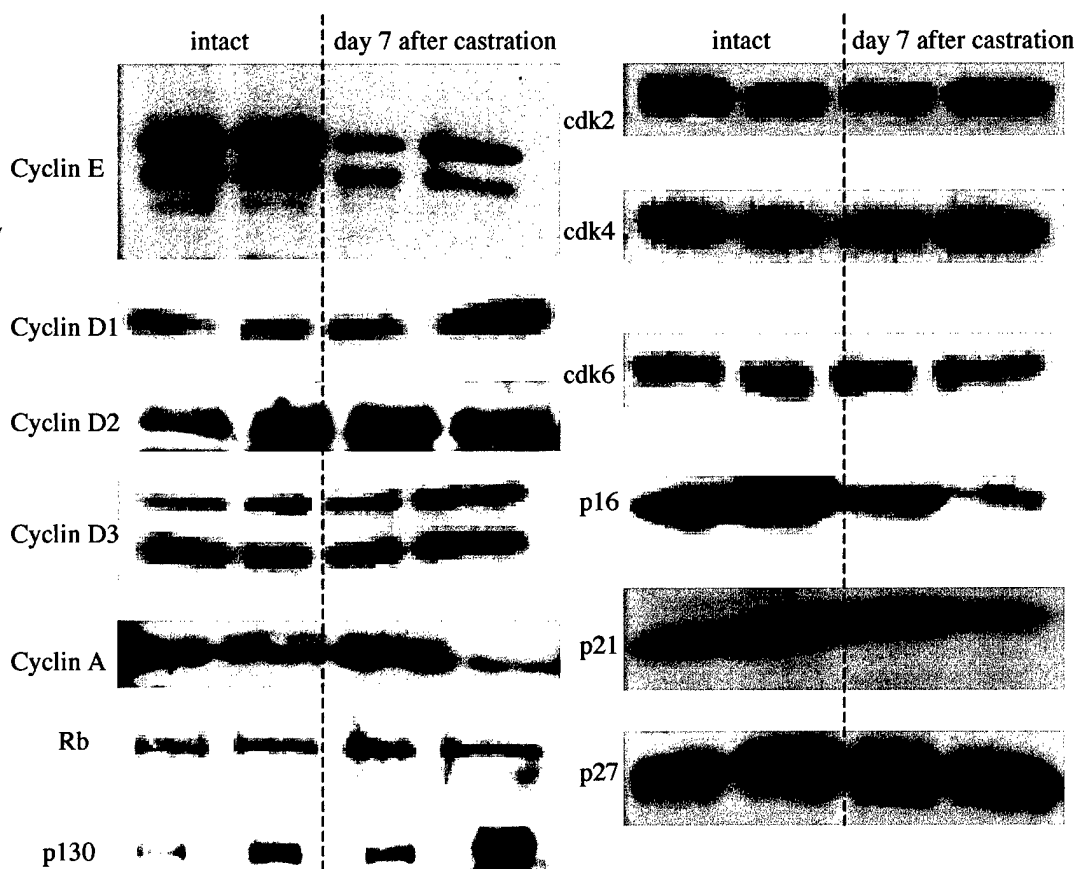


Fig. 2. BrdU staining of MDA PCA 2b tumor tissue fixed in formalin. Dark brown nuclei are considered BrdU-positive, magnification: 60x. Tumors were taken from (A) an intact mouse, (B) a 3-week-castrated mouse treated subsequently with vehicle for 5 days, (C) a 3-week-castrated mouse subsequently s.c. injected with 8.6 mg testosterone propionate/kg body weight for 5 days.

Fig. 3. Western blot analysis of cyclin, cdk and cell cycle inhibitor in MDA Pca 2b tumors taken from intact mice and from castrated mice at day 7 after castration. Tumor protein lysates were electrophoresed and transferred onto a nitrocellulose membrane. Antibodies against the proteins indicated were used in western blot analysis.



Research Accomplishments

MDA Pca 2b tumors were isolated from intact tumor-bearing mice and from tumor-bearing animals at various time points after castration and subsequent androgen replacement. Tissue was fixed in formalin for (immuno)histo-chemistry and the remainder was snapfrozen for biochemistry. Hematoxylin-and-eosin staining showed that the morphology of MDA Pca 2b grown in nude mice resembles a poorly-differentiated prostate tumor (Fig. 1).

Mice received BrdU 1 hour before sacrifice and tumor tissue of each mouse was fixed in formalin. Immunohistochemical staining of tumor tissue for the presence of BrdU showed that cell proliferation in MDA Pca 2b is androgen-sensitive: BrdU incorporation decreases upon castration and increases following androgen replacement (Fig. 2). No effect of androgen deprivation on apoptosis was seen: intact tumor tissue and tumor tissue obtained 7 days after castration subjected to the TUNEL assay displayed a similar, low rate of apoptotic cells. In addition, long-term castration (up to 3 months) appeared not to affect the size of the Pca 2b tumors. Though tumor regression occurred in the CWR22 human prostate cancer xenograft model upon androgen withdrawal, changes in apoptosis were not observed at any time after androgen withdrawal in CWR22 (Agus et al., 1999) similar to our findings with MDA Pca 2b.

Initially, western blot analysis of various cell cycle proteins was performed on proteins isolated from tumor tissue of intact and 7-day castrated mice. The results are shown in Fig. 3; the levels of cyclin dependent kinases 2, 4 and 6 appeared to remain unchanged 7 days after removal of androgen. Similarly, no change was observed in the levels of D-type cyclins and cyclin A 7 days after castration. Levels of cyclin E decreased in the absence of androgen. Of the cell cycle inhibitors, no change was detected in the levels of p21 and p27 7 days after castration, while p16 levels decreased after androgen removal. The levels of Rb and p130 were not affected at 7 days of androgen ablation.

Next, proteins isolated from tumor tissue taken at various time points after castration (days 1 to 10) were subjected to western blot analysis of cyclin D1 and cyclin E. Proteins isolated from tumors collected at days 1-38 after castration and at days 1 to 5 after start of androgen replacement were analyzed for the expression of p27 and c-myc by western blot.

Preliminary results show that following castration of mice carrying MDA Pca 2b tumors the level of cyclin D1 increases on day 1, decreases on day 2 and subsequently increases from day 3.5 until day 5 after which there is a drop until control levels at day 10 (Fig. 4a). The levels of cyclin E appear to decrease following castration from day 1 up until day 10 (Fig. 4b). More samples need to be assayed to determine whether a slight increase occurs on day 10, but the level remains far below the intact level. The level of the cell cycle inhibitor p27, which was found not to change at day 7 after castration, appears to drop down at day 10 after castration (Fig. 5a). Following androgen replacement p27 levels gradually increase and reach a maximum, which is still below the intact level, after 3 days on TP (Fig. 5b). Five days on TP causes the p27 level to drop till background levels (Fig. 5b). We were able to detect c-myc, a labile protein, in protein samples that were kept overnight at 4° C but not in frozen protein samples. Levels of c-myc decrease after castration (Fig. 6). So far, samples of castrated mice treated for 1 and 2 days with TP have not been tested, but androgen replacement for 3 and 5 days appears not to affect the levels of c-myc which remain at (or possibly return) to the castrated, background level (Fig. 6).

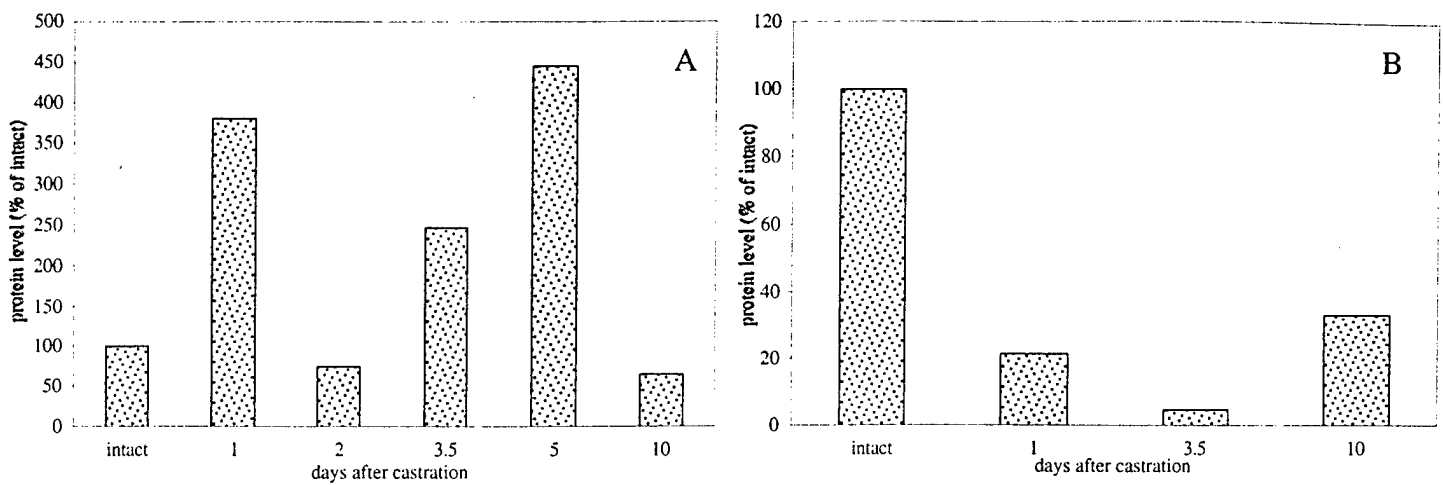


Fig. 4. A: cyclin D1- and **B:** cyclin E-expression in MDA Pca 2b tumors taken from intact mice and from castrated mice at various time points after castration. Tumor protein lysates were electrophoresed and transferred onto nitrocellulose membrane. Specific antibodies were used in the western blot analysis. Bio-image analysis was used to quantitate the protein expression levels.

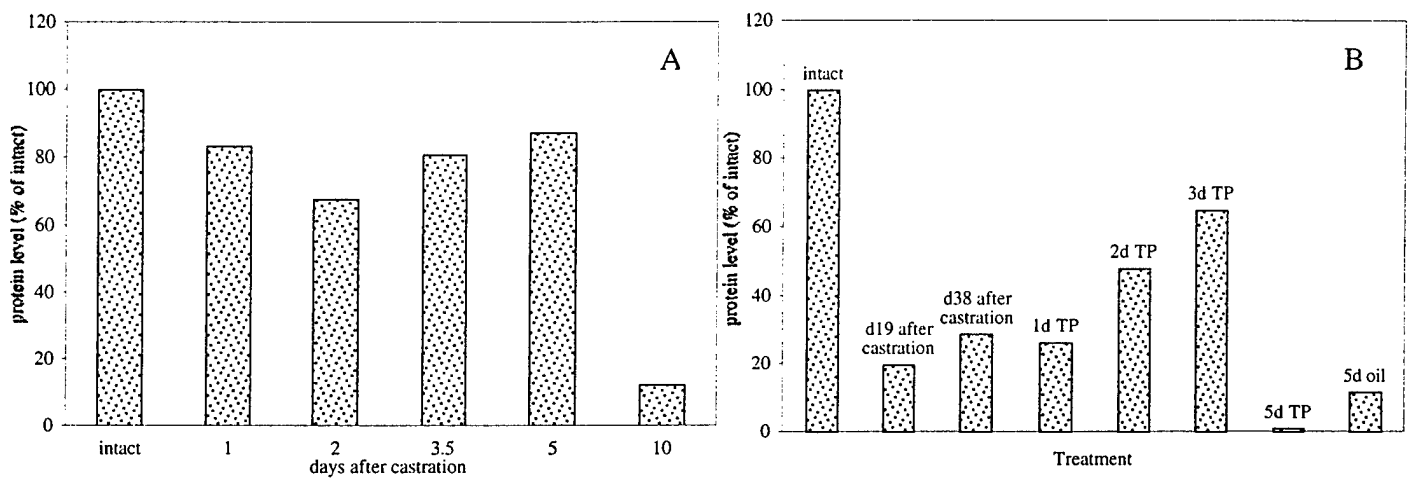


Fig. 5. P27-expression in MDA Pca 2b tumors taken from intact mice, from castrated mice at various time points after castration and from 3-week-castrated mice that were treated with testosterone propionate (TP) for various days. Western blot analysis and quantitation were performed as described in the legends of Fig. 4.

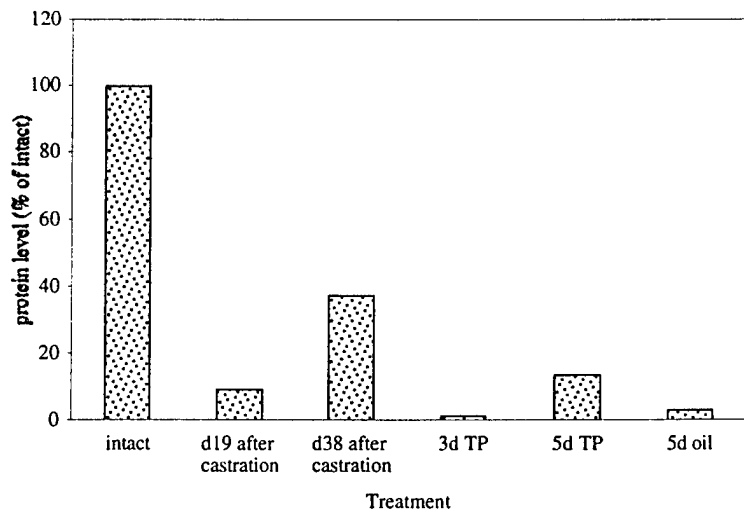


Fig. 6. C-myc expression in MDA Pca 2b tumors taken from intact mice, from castrated mice at various time points after castration and from 3-week-castrated mice that were treated with TP for various days. Western blot analysis and quantitation were performed as described in the legends of Fig. 4.

Earlier our laboratory showed that androgen replacement in castrated rats induces D-type cyclins and cyclin E at various time points during androgen replacement (Chen et al., 1998). In castrated rats a low p27 level was detected which dramatically increased after androgen replacement, with induction levels being less at the peak of prostate proliferation and higher when proliferation was low (Chen et al., 1998).

Recently, cell cycle alterations following castration were detected in the xenograft CWR22 using immunohistochemistry (Agus et al., 1999). In contrast to western blot quantification of immunohistochemical techniques is subjective, but allows for study at the level of the cell. The authors showed an upregulation of p27 up until day 7 after castration after which p27 levels remained stable while cyclin D1 levels increased up until day 10 after castration before stabilizing (Agus et al., 1999). The difference between these and our results are not surprising as prostate cancer is known for its heterogeneity.

In the approved proposal our central hypothesis was that dissociation between androgen-induced proliferation and androgen induction of p27kip1 is one of the mechanisms involved in prostate cancer. Furthermore, we hypothesized that in androgen-dependent tumors testosterone induces cyclin D1 and downregulates p21 without changing the expression of p27kip1. We postulated that androgen withdrawal in these tumors will result in downregulation of cyclin D1 and upregulation of p21. We observed downregulation at day 10 after castration while p21 levels were unchanged at day 7 after castration. Following androgen withdrawal we observed p27 downregulation and p27 appeared to be induced by androgen replacement. In our hypothesis, we did not take into account a change in a cell cycle inhibitor of the INK4 family, whereas we did detect a decrease in level of p16. This inhibitor is specific for cdk4/6 which complex with D-type cyclins. The p16 decrease may be a result of a decrease in the levels of cyclin D1. Likewise, the downregulation of p27 at day 10 may be related to the downregulation of cyclin E, one of the targets of p27. Based on our results a redefining of our hypotheses appears necessary. Recent publications show that p27 does not only act negatively on the cyclin E-cdk2 complex thereby preventing cell cycle progression. In addition, p27 acts positively on complexes of D-type cyclins and cdk4/6: sequestration of p27 in cyclin D/cdk complex lowers the inhibitory threshold and facilitates activation of cyclin E/cdk2 (reviewed by Sherr and Roberts, 1999). In addition, p27 is regulated directly by cyclin E/cdk 2 and indirectly by c-myc (reviewed by Elend and Eilers, 1999). The p27 decrease often observed in prostate cancer appears to be a result of increased ubiquitin-mediated degradation. The cyclin E/cdk2 regulation of p27 involves phosphorylation on Thr 187 thereby targeting the protein for degradation. Our redefined hypothesis states that p27 is abnormally regulated in prostate cancer; following androgen withdrawal c-myc levels will decrease more slowly relative to p27 and a high cyclin E/cdk2 activity will be maintained. Androgen replacement will induce an increase of c-myc and cyclin E which will counterbalance the androgen-induced increase in p27 levels, resulting in high cyclin E/cdk2 activity.

Deviations from original Statement of Work

The original Statement of Work was based on the availability of androgen-dependent transferable human prostate tumors. Studies were started with MDA Pca 2b provided by our collaborator Dr. Navone. However, a very recent publication shows that in MDA Pca 2b the androgen receptor (AR) is doubly mutated: 2 missense mutations in the ligand binding domain change leucine at position 701 to histidine and threonine at position 877 to alanine (Zhao et al., 2000). As a result the Pca 2b AR has a 50-fold reduced affinity for testosterone relative to wild type AR and a high-affinity for cortisol/cortisone such that the physiological concentration of free cortisol and cortisone greatly exceeds the binding affinity and would activate this mutated AR (Zhao et al., 2000). Thus, MDA Pca 2b although androgen-sensitive cannot be considered androgen-dependent and Tasks 2 and 3 of the Original Statement of Work cannot be performed using MDA Pca 2b. The androgen-sensitivity makes this xenograft still useful for Task 1 and this task will be completed. Since our collaborator has available MDA Pca 2a and MDA Pca 2b which both possess the doubly mutated AR, we would like to propose to perform Tasks 1 and 3 with a transferable human prostate tumor LAPC9 which has been described (Craft et al., 1999). This tumor is androgen-responsive and expresses the wild type AR. Tumors cease to grow following castration of tumor-bearing mice and remain dormant for at least 6 months, after which finally spontaneous, androgen-independent tumors reappear. We have asked permission from Dr. Sawyers (UCLA Jonsson Cancer Center) who developed this xenograft (see enclosure).

We believe that in the remaining funding period Task 1 can be completed for the xenograft MDA Pca 2b since all necessary samples have been collected. Completion of Tasks 1 and 3 but not 4 are realistic for xenograft LAPC9. Completion of Tasks 1 and 3 for LAPC9 will give insight in the cell cycle alterations associated with transition to androgen-independence of this androgen-dependent prostate tumor.

Key Research accomplishments:

- * Proliferation of MDA Pca 2b tumors has been shown to decrease in absence of testicular androgen and to increase upon androgen replacement.
- * Apoptotic rate in MDA Pca 2b tumors has been shown not to be affected by the absence of androgen
- * Levels of cell cycle proteins, i.e. cyclins D1, E, A, p27, p21, p16, Rb, p130, c-myc have been determined in MDA Pca 2b tumors from intact and castrated mice
- * Conditions for detection of c-Myc by western blot have been established
- * Conditions for detection of small cell cycle proteins (p21, p16) have been improved.
- * Levels of cell cycle regulators p27 and c-myc upon androgen replacement have been determined.
- * MDA Pca 2b tumors do not regress upon long-term (up to 3 months) castration.

Reportable Outcomes

None

Conclusions

-Training: The involvement in grant writing has led to greater understanding of this process. Based on data obtained with the training funding the trainee will write a grant proposal for the Internal Research Grant Program, an intramural grant funding program at UT-MDACC, Houston. Attending the Gordon Conference and involvement with the Science Park mouse prostate cancer model has shown the trainee the limitations and the benefits of the human prostate cancer xenograft model.

-Research: Our results as well as recent reviews on p27 functioning in the cell cycle show that our original hypothesis that androgen-induced proliferation and androgen-induction of p27 are dissociated in prostate cancer needs to take into account the diverse regulators of p27. Our refined hypothesis states that in prostate cancer p27 is abnormally regulated resulting in a high level of cyclin E/cdk2 activity which induces G1 to S phase progression. Secondly, very recently it was published that MDA Pca 2b which we used as a model for androgen-dependent prostate cancer has a doubly mutated androgen receptor. Thus, though androgen-sensitive, MDA Pca 2b turns out to be androgen-independent and, moreover, to have a high affinity for cortisol/cortisone.

We will complete our cell cycle studies with MDA Pca 2b which so far have given us insight into the complexity of p27 regulation in prostate cancer. Cell cycle studies will be started in the human prostate cancer xenograft model LAPC9 which has been shown to be androgen-dependent and to have a wild type androgen receptor. Cell cycle studies of LAPC9 and its androgen-independent counterpart which has been reported to grow spontaneously after at least 6 months of castration of LAPC9 tumor-bearing mice will clarify cell cycle alterations associated with the progression to androgen-independence of prostate cancer.

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Appendices

None